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A quantitative study of the morphology of normal, hyperplastic, and neoplastic mammary tissue in the mouse

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A QUANTITATIVE STUDY OF THE MORPHOLOGY OF NORMAL, HYPERPLASTIC, AND NEOPLASTIC MAMMARY TISSUE IN THE MOUSE

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To Dr. Katherine Knapp, Dr. Alice Hunter, Dr. F. M. Nahhas, Orlando and Edna Hill, and Elizabeth Rathman.

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INTRODUCTION

Mouse Mammary Tissue.

Gross Morphology. The mouse has five pairs of mammary glands, three thoracic and two inguino-abdominal. In the female the glands undergo changes associated with estrus, pregnancy, lactation, and involution. The glands in the male remain rudimentary (40).

Microscopic. The nipple of each mammary gland consists of three epidermal layers: the stratum germinativum, the stratum granulosum, and the stratum corneum which is covered by an elastic epidermis. A single major duct in the stratum germinativum leads into the subcutaneous fat pads, where it forms a duct system. None of the duct systems are connected to any others (11, 19, 28).

Embryology. The development of the mouse mammary gland begins during fetal life with the formation of a ductal system by tubular invagination of the germinal layer of epidermis (22). The first evidence of mammary glands is seen in the 10-11 day embryo with the appearance of mammary gland streaks. These streaks are the result of the proliferation of the Malpighian layer and convert into paired mammary lines. By the thirteenth day, the hillock stage of the mammary bud development is observed at intervals along the lines. In the

fifteen day embryo, the buds round off and continue to invaginate. By the eighteenth day, a primary sprout (anlage) begins to form from the distal end of the bud. The secondary sprouts start to form by the time of birth. The epithelial ingrowths which form the nipple appear and sink into the mesenchyme for a period after birth (40, 45, 46).

Development. From the time of birth to the end of the first week, the secondary sprouts continue to grow and form ducts and the tertiary and quaternary sprouts begin to form. From the beginning of the second week until puberty (approximately 6-8 weeks in the female mouse), the glands grow slowly both in length and number of ducts (10).

Turner (45) examined whole mounts of mammary glands at various stages and found that a principal (primary) duct comes from the nipple which branches to form a pair of secondary ducts. In the early stages of growth, the ducts undergo a regular dichotomous type of branching. Each duct spreads out in a single plane surrounded by circular connective tissue fibers which eventually become the gland stroma. As growth progresses, new lateral branches sprout irregularly from the chief ducts. In some cases secondary branches develop between the nipple and the bifurcation of the chief ducts. At the distal end of each duct or sprout are sac-like structures composed of a solid mass of epithelial cells which are points of active growth of the duct system and which Turner termed "end-buds" (45).

The cuboidal epithelium along the length of the ducts is one-layer thick. The ducts at their ends have a cuboidal to low columnar epithelium which has a tendency to establish a double layer. Within the cells there exists a row of large oval nuclei with scattered chromatin. The cell boundaries are not very distinguishable and the cytoplasm is granular (6). The connective tissue envelope is well marked. Mitosis is rare and is not related to the phase of the estrus cycle (9). The growth and change associated with each successive estrus consists of changes within the existing cells in the epithelium of the duct endings rather than a sudden burst of mitosis resulting in multiplication (13, 41).

Macroscopic Changes

Inactive. Various macroscopic studies have been done on the mammary gland of the virgin female mouse during the cyclical series of hormonal changes (Figure A). It has been shown that during diestrus, a short period of sexual quiesence occurring between metestrus and proestrus in female mammals, the glandular parenchyma consists of an open network of narrow ducts with a small number of simple branches which are scattered throughout the stroma (7, 9, 10, 13, 23). The buds at the ends of the ducts consist of a solid mass of epithelial cells. The extent to which the ducts spread through the pads of subcutaneous fat which surround the mammary glands is controlled by age. The lumen of the duct adjacent to the end

bud is wider than at other parts of the duct except in places where branches occur. Development occurs with the onset of the first estrus with a slight burst of growth at each subsequent estrus[1] (24, 37).

During proestrus, the period of heightened follicular activity preceding estrus in female mammals, light staining buds appear on the ducts particularly around the periphery of each gland, and marked signs of growth occur in the region of the nipples where a large number of blunt projections appear on the main ducts. Toward the end of proestrus, the endings of the ducts which are characteristic of early estrus begin to appear around the periphery of the glands further from the nipples (27, 37).

During estrus, the recurrent, restricted period of sexual receptivity in female mammals other than human females marked by intense sexual urge, the mammary ducts become dilated and distended with fluids. The buds formed during proestrus become elongated and stain deeply. The ducts are relatively free from small projections with the network of glandular tissue becoming denser. Regression sets in before the termination of estrus which generally lasts two days (9, 10).

During metestrus, the period of subsiding follicular function or rest following estrus in female animals, the

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[1]The growth of the duct system during the prepubertal period is only sufficient to keep pace with general body growth. At the approach of puberty, however, the mammary ducts begin to grow rapidly. Puberty is usually reached during the 8th or 9th week (27).

ducts decrease in width. Projections at the ends of the ducts disappear by late metestrus, the collapsed duct endings having an irregular outline. By the end of metestrus the ducts have returned to a condition slightly in advance of the previous diestrus (24, 27).

The mammary gland duct system becomes slightly extended during each estrus cycle after reaching puberty. After conception, however, instead of the slight regressive change which normally takes place when pregnancy does not occur, the mammary glands begin a new phase of growth (9).

Pregnancy. There are two phases of development of the mammary glands during pregnancy (Figure B). During the first half of pregnancy the duct system rapidly sprouts a lobule system. By the middle of pregnancy, the process of cellular secretion is initiated, which causes an expansion and unfolding of the alveoli until, at the approach of parturition, the secretory process has reached a high level with the entire gland engorged with secretion (22, 39, 43).

Upon impregnation, growth begins at the margins of the separate glands (6). This growth is different from the growth of the buds which sprout new branches of the duct system in virgin glands. It differs both in position and morphology. Dilation at the ends of the ducts occurs first and dilation of the entire duct second. Buds begin to develop along the length of the smaller ducts. Pads of subcutaneous fat decrease with the increase of glandular tissue (7).

Six days after copulation active budding and formation

of alveoli occur along the length of all ducts. The buds representing the anlage of the lobes of the gland increase in length to form the intralobar duct of the future lobe. At the terminal end and lateral walls of this duct appear secondary bud out-growths in the gland of a ten day pregnant mouse. (From three to five of these buds may be observed at the end of such an intralobar duct.) These buds develop into short interlobular ducts along the ends of which more buds appear. These latter buds become the alveoli (46).

Growth takes place in one direction at right angles away from the midventral line. As a result of this growth process, the nipple lies outside the main glandular mass until the end of pregnancy. It then becomes partially enclosed by the hypertrophy of the adjacent lobules (7, 40).

The mammary gland of pregnant mice has been studied at different days and therefore different stages of development (7, 10, 22). By the twelfth day of pregnancy, growth and budding proceed. A large number of alveoli have developed along the length of all ducts. The total area and density of the glandular tissue has increased. At this time the hyperplasia of the gland parenchyma is complete.

Individual glands have met and interlocked in the midventral line around the anus and for a short distance up the abdomen; also in the mid-dorsal line along the shoulders and the back of the neck. The first thoracic gland grows forward and upward in front of the insertion of the forelimb while the second and third thoracic ducts grow slightly

backwards and upwards eventually meeting behind the forelimb (46).

By the fifteenth day of pregnancy, clumps of alveoli are packed along all ducts with the glandular tissue replacing fat and connective tissue. The network of glandular tissue becomes progressively more dense until approximately the eighteenth day of pregnancy. The ducts at this point are no longer evident and the lobules can be distinguished only at the periphery (39).

Lactation. After parturition, growth continues for six days. All visible distinctions between neighboring ducts and their branches are lost with the compact ducts and alveoli interlocking. The main ducts have become dilated and distended with secretory fluid and the entire glandular mass has a thickened even appearance. Glandular parenchyma predominates reaching its maximum development at twelve days post-partum.

Twenty-one days after parturition (when weaning normally occurs in the mouse), the mammary glands are becoming inactive - - no longer compact but having the appearance of a dense network thinning out toward the edges. Several of the separate glands cease to secrete. When the litter is small (2-3 pups), two or more glands may be neglected and allowed to regress (7, 9, 10, 14, 23, 25, 34, 38, 45).

Involution. Following weaning there is an accumulation of milk in the gland for 24 hours or more because the retained milk inhibits further secretory activity (25). By

the fifth day the gland has lost much of the milk from the alveoli and ducts which appear much thinner than during lactation (34).

General contraction of glandular tissue takes place. At the periphery of the gland and around the nipples, the separate branches can be distinguished. The alveoli are small and irregular but still numerous (46).

At six days post-weaning, regression and further reduction are occurring in the area with decreasing thickness of the individual glands. At nine days, the regression has become rapid with collapsed ducts and degenerating alveoli.

Regression is complete by the thirteenth day. The ducts are reduced to a thread-like condition with a thin layer of cells. There is a further decrease in area and thickness of the glandular tissue. The separate ducts and lobules can be distinguished without difficulty. In comparison to the gland of the unmated mouse, the network of the parenchyma is considerably denser with its ducts projecting numerous short branches (7, 23).

If pregnancy does not occur following ovulation, the glands undergo slight retrogressive changes; however, as the next estrus approaches, the glands again show evidence of proliferation. With each succeeding estrus cycle the growth of the ducts is slightly extended. These proliferative changes are more obvious during the first few cycles than later when the growth of the duct system is more extended (46).

Hyperplastic Alveolar Nodules (HAN).

Hyperplastic alveolar nodules (HAN) occurring in strains of mice susceptible to mammary cancer are generally considered precancerous, that is, prone to undergo neoplastic transformation.

DeOme, Faulken, Bernard and Blair provided direct experimental evidence which showed that the hyperplastic alveolar nodules gave rise to mammary tumors (17). In their work, a series of three experiments was conducted in which normal, nodular, and tumorous mammary tissue was transplanted into host mammary fat pads. Subsequent growth patterns were studied since normal, nodular, and cancerous mammary tissue produces different types of growth patterns. Neoplastic transformation (mammary adenocarcionoma) occurred in over 50% of the nodular transplants whereas less than 1% neoplastic transformation occurred in the normal transplants.

Spontaneous abnormal proliferation of the mammary epithelium occurs frequently. The degree to which it develops varies with some hereditary factors (44).

No structural factor is associated with the intrinsic hereditary predisposition toward mammary carcinoma in mice (27). The hereditary factor seems not in the gland itself but rather in the hormonal stimuli that accompany normal development (31).

Various hormonal, cytologic, histochemical, radiographic, and macroscopic studies comparing the HANs with mammary tumors and with normal mammary gland tissue have

demonstrated that the HANs resemble normal mammary gland tissue more than mammary tumors (5, 24). The typical HAN consists of an area of alveolar hyperplasia resembling a normal lactating mammary lobule, occurring however, in an inactive mammary gland (2, 3, 12, 18, 26, 16).

A summarization of the characteristics of HANs in mice has been made:

1. the HAN is more common in strains which have a high incidence of mammary cancer than in those with low incidence;
2. the nodules increase in number with age;
3. the nodules show variable degrees of independence from the hormones that support and maintain normal mammary gland growth and development;
4. the HANs are lobuloalveolar;
5. the HANs are large enough to be seen at low power (2-4X) under a dissecting microscope; and
6. they can be arranged in an arbitrary morphologic sequence from normal to carcinoma in situ (32, 33, 47).

DeOme, Bern, Nandi, Pitelka and Faulkin characterized hyperplastic growth into three general morphologic categories, the most common type being a reasonable replica of a normal mammary gland during late pregnancy except that the elements are more densely packed together. A second type of hyperactive growth is a slowly expanding, densely packed, alveolar structure which DeOme termed as "expanding nodule". The third type of nodular growth is a replica of the host's glands, and is composed of ductal elements; but it usually can be differentiated from a normal gland by minor structural

irregularities. A gland will usually exhibit many variations and combinations of these growths (18).

Transplantation studies (3, 4, 15, 21, 29, 35, 36) suggest that the appearance of a nodule represents the growth of an altered population of cells, and that the appearance of a tumor represents the subsequent occurrence and growth of another altered population of cells.

This transformation of HAN growth into neoplastic tissue is the basis for its identification as a premalignant lesion.

Purpose. Numerous studies have been conducted to relate hormonal levels to mammary morphology and mammary neoplasia (6, 13, 14, 15, 16, 18, 24, 29, 31, 43, 44). Relatively few studies, however, have examined the mammary gland morphology of the normal mouse in various functional states. A logical approach to an expanded understanding of mammary tumor morphology is an examination of normal then premalignant and finally malignant mammary tissue.

This study intends to provide a quantitative and qualitative histological account of two aspects of mammary gland morphology. First, the normal mouse during different functional phases including inactivity, pregnancy, lactation and involution is presented. Second, the abnormal changes of mammary hyperplasia and neoplasia - both spontaneous and exogenous - are examined.

MATERIALS AND METHODS

Characterization of Tissue.

Eight C_{57}BL/6J Jackson Laboratory hybrid female mice of approximately one year (genetically similar to the C_3H/HeJ strain) were used. Four stages of development of normal mammary tissue were studied:

1. inactive or resting virgin;
2. pregnant female (late pregnancy approximately 17 days);
3. lactating animal (pups 2-3 days old); and
4. involuting (pups 17-20 days old).

Five C_3H/HeJ Jackson Laboratory hybrid female virgin mice approximately one year old were studied specifically for their ability to spontaneously develop mammary adenocarcinomas and accept and subsequently grow syngeneic exogenous neoplastic mammary tissue. Five neoplastic stages were included in the study:

1. a spontaneous tumor approximately 0.5 cm in diameter;
2. the first passage of the spontaneous tumor;
3. the second passage of the spontaneous tumor;
4. the first passage of a syngeneic exogenous tumor (H 2712)[1];

[1]Provided by Dr. Arthur Bodgen, EG&G Mason Research Institute, Worcester, Massachusetts 01608.

5. the third passage of the preceeding syngeneic exogenous tumor.

These nine tissue samples constitute the categories studied (Table 1).

Biopsy of the Normal Tissue.

Under sterile conditions, the mice were pretreated with 0.1cc atropine sulfate (0.5N) administered subcutaneously. After ether anesthetization, the mice were placed in a supine position. The abdominal area was cleansed with 70% ethanol and a small area of fur around the right bottom inguinal nipple was shaved. A shallow incision was made cephalad (0.5 cm) from the nipple. The skin was gently pulled back to expose the white pulpy mammary tissue and a piece, approximately 1.0 cm in diameter was excised, washed with normal saline (0.85%) and deposited in alcohol-formalin-acetic acid fixative. Two to three nylon sutures (4/0) were used to close the site of biopsy.

Transplantation Technique.

All equipment used was sterile. The donor was sacrificed and the area surrounding the tumor cleansed with 70% ethanol. A midline incision was made through the skin where the tumor was implanted. The flap of skin was pinned back exposing the tumor mass. The tumor was freed with scissors, washed with sterile saline (0.85%) and transferred to a sterile Petri dish placed over ice. Necrotic material was removed. A piece of tumor (approximately 1.0 cm in diameter) was deposited in fixative for later histologic study. The rest of

the tumor was cut with a sterile razor blade into cubes, approximately 2 x 2 x 2 mm. A thirteen gauge trocar was washed with a sterile balanced salt solution (Earle's). With forceps, a fragment of tumor was placed into the tip of the trocar. The animal was anesthetized with ether until motor coordination was lost. The fur and skin around the area of the implant site was swabbed with 70% ethanol. The skin was pierced in the inguinal area with the tip of the trocar and pushed forward subcutaneously to the axillary area. The tumor fragment was ejected from the trocar by pushing the plunger as far into the barrel as possible. The barrel and plunger were turned and removed. No suturing was necessary. This procedure represents a modification of the procedure recommended by EG&G Mason Research Institute (20).

Histologic Preparation of the Mammary Tissue.

The procedure for dehydration, clearing, infiltration and embedding with paraffin, sectioning, mounting, and staining described by Humason was followed (30). Seven micron serial sections were cut, mounted, stained with hematoxylin and eosin, and dried for 24 hours. Twenty-six slides were prepared; twelve were used for photographs. Certain subsequent sets (neoplastic tissue in particular) were processed by Technicon tissue processor. Six slides were prepared and thirty-six photographs taken.

Photography.

Photographs of the first twelve sections were taken with

a Pentax camera at 10 power (for 1/125th sec) and 40 power (for 1/30th sec) using a Zeiss microscope. High contrast, fine grain film (Ilford HP5) and paper (Kodak #5) were used for enhanced contrast of tissue. Ten frames at 10 power were taken for each of the twelve slides, or 120 frames. Two frames at 40 power were taken for each slide for a total of 8 frames. From these 128 frames, 5" x 7" pictures were developed and separated into each of the four categories (inactive, pregnant, lactating and involuting). Three pictures were selected (turned upside down and picked at random) from each group. The twelve resulting pictures were used for the statistical evaluation of the mammary tissue at the four stages.

Photographs of the six neoplastic tissue slides were taken with a Cannon camera at 10 power (for 1/60th sec) using a Leitz microscope. Thirty-six pictures (six per slide) were taken and developed. The selection process was the same as that previously mentioned. Fifteen pictures were used for the statistical evaluation of the neoplastic tissue.

Statistical Evaluation.

Two transparent grids (4 squares per inch and 8 squares per inch) were superimposed on each photograph. The underlying tissue in each square was categorized as epithelial or nonepithelial according to its contents (Table 2). For the neoplastic tissue, three categories were included: epithelial, nonepithelial and necrotic. Summation of the

number of squares in each category allowed development of a ratio of epithelial to nonepithelial cells for each photograph. Necrotic tissue was not counted. This was done for both the 4-square and 8-square grid. A mean ratio of epithelial/nonepithelial tissue was determined for each of the nine categories. The significance of the difference between nonepithelial/epithelial ratios in the various categories was determined using the Fisher Exact Probability Test (42). A p-value of less than or equal to 0.05 was considered significant.[3]

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[3]The Fisher Exact Probability Test is a nonparametric technique for analyzing discrete data (either nominal or ordinal) when the two independent samples are small in size. It is used when the scores from two independent random samples fall into one or the other of two mutually exclusive classes. The scores are represented by frequencies in a 2 x 2 contingency table. The test determines whether the two groups differ in the proportion with which they fall into the two classifications. (42) (Addendum I)

RESULTS

Normal Mammary Tissue.

Gross Anatomy. The mouse mammary gland was spread over a wide area of the anterior body wall and appeared to lie almost in a single plane of equal thickness. The healthy tissue was white in color, opaque and pulpy in texture.

Microscopic Anatomy. In the inactive mammary gland section, adipose tissue was abundant and glandular material minimal - there were few alveoli. It was difficult to distinguish between alveoli and ducts since lobes and lobules were not well defined. Little secretory product was evident; blood vessels were dilated.

A biopsy of mammary tissue taken from the pregnant female revealed distinct changes; there was a development of the duct system with an increase in secretory alveoli. Previously the alveoli were compact, deeply staining structures of spherical or cuboidal shape and slightly irregular form. During pregnancy, the alveoli had increased in size, become more irregular and stained less deeply. With this enlargement of alveoli, relatively smaller amounts of grandular stroma were evident. Lobules were easily identified since they were compressed and expanded with alveoli. The presence of lipid which was dissolved by the fixation process was apparent in the secretory cells. Adipose tissue

was predominant with some secretory product (precipitated protein) in ducts. At this stage, there was extensive capillary network throughout the tissue.

Three histologically similar fluids were evident - secretory product produced by the alveoli, lipid (in the lumen and in the secretory cells), and plasma. The plasma was restricted to the veins and arteries, staining pink with hematoxylin and eosin; the secretory product also stained pink and was retained in the ducts; because of the fixation process, the lipid areas were clear and were located within the secretory cells, ducts, and alveoli.

In the lactating female (pups 3 days old) lobes and lobules were well defined and packed with secretory alveoli. The tissue was almost entirely grandular - the alveoli in different stages of activity as shown by various sizes and irregular shapes. Some cells were large and filled with pink staining material; other cells appeared decreased in size (due to the secretory product in their apical portion being released). At this stage there was very little adipose tissue - the majority of the tissue being epithelial cells.

Tissue in the involuting stage (pups 17-20 days old) was regressing almost to the inactive state. A few alveoli remained with a small amount of secretory product; however, most of the alveoli had disappeared, lobules had shrunk in size and there was a reappearance of the adipose tissue.

Neoplastic Mammary Tissue.

When the spontaneous tumor was excised, the tissue lacked the smooth, shining, pure white appearance of the healthy tissue. The hard, encapsulated tumor was a pasty color and oozed a milky fluid. After each successive passage, the tumor darkened, become more crenated and vascularized. The capsule surrounding the tumor was retained throughout the passaging and the tumor maintained a definite oval shape.

The exogenous tumor was irregular in shape, tan and highly vascularized. The tumor was soft, honeycombed with cavities and not encapsulated. With each successive passage, the syngeneic transplant contained increasing amounts of necrotic tissue (grey in color), became more irregular in shape, more vascularized around the edges with less vascularization within the tumor and with abundant connective tissue.

The rate of growth of the C_3H spontaneous carcinoma was very slow - barely palpable to 0.5 cm in diameter in two months. The C_3H syngeneic transplant grew rapidly - approximately 1.75 cm in diameter in 24 days.

Necrosis and inflammation were absent in the small spontaneous tumor in its first and second passages but was present in the just palpable exogenous tumor. Necrosis was rarely seen in the spontaneous tumors even when they were large.

The exogenous tumor was consistently more vascularized then the spontaneous tumor. The relative amount of other nonepithelial tissues, mononuclear cells, fibroblasts and

interstitial space was higher in the exogenous tumor than spontaneous. Approximately one-third of each section of exogenous tumor examined was fibrous connective tissue and necrotic tissue. There was no consistent change in the passaged spontaneous tumor.

The cancer cells of the spontaneous tumor grew in numerous tightly spaced glandular-tubular structures similar (as mentioned previously) to those of a normal lactating gland. The cells in the exogenous tumor, also high in number, lacked however, a definite shape or arrangement. The individual exogenous cells were moderately pleomorphic and had slightly enlarged irregular dyskaryotic neuclei. In the spontaneous tumor, blood vessels were in the internodular tissue and within the nodules (i.e. normal blood supply); in the exogenous transplants vessels were confined to internodular areas only. Centrinodular necrosis was present in the exogenous but absent in the spontaneous tumor. Viable tumor cells were well differentiated and formed acini only in the spontaneous tumor and were poorly differentiated in the exogenous tumor. The other nonepithelial tissues increased with each passage of the exogenous tumor while vascularization decreased.

The ratios resulting from separating the various tissue samples into epithelial/nonepithelial categories using a 4 sq/in grid are listed in Tables I, II, and III, and Figures I, II, and III.

DISCUSSION

Normal Anatomy.

Gross and Microscopic Anatomy. In this study, the morphological findings in normal tissue in all stages of functional development agreed with previously cited reports (7, 9, 10, 11, 15, 23, 27, 34, 37, 40, 45, 46). As expected, the differences in the gross appearance of the exogenous and spontaneous tumor were reflected by changes at the histological level. Gross observable differences included color, texture, vascularization, presence of necrotic material, presence of a capsule, and invasiveness. These grossly observable differences were found to be reflected in comparable histological changes.

Richardson described the morphology of mammary glands as having a parenchyma made up of a system of ducts whose terminal ducts expand into acini or alveoli. A group of alveoli were said to form a lobule encircled by connective tissue. The alveoli that made up the lobuloalveolar system were defined as small vesicles of unequal size (37). This study has extended the qualitative description by Richardson to a more detailed quantitative analysis that includes measurements of the changing normal mammary gland, the precancerous mammary lesion, and tumor tissues of two different origins. Because the results agree using a quantitative model for the

level of function comparisons can be made with an objective numerical basis.

Five morphological changes occurred with increasing activity in the normal tissue:

1. an increase in the number of functioning epithelial cells,
2. a decrease in the number of fat cells,
3. a decrease in the relative amount of connective tissue,
4. a decrease in the size of the fat cells, and
5. an increase in the amount of lumen space.

As this study progressed it became apparent any of these parameters could be used to quantitatively monitor the level of activity of normal tissue.

During pregnancy, the secretory product in epithelial cells became more profuse resulting in precipitated protein in tubular lumens on section. Growth is reported to continue until the 12th day of lactation when gland development reaches its peak (10, 40). This concurs with findings of this study--the raio of epithelial/nonepithelial being the highest in the lactating female.

Involution in the mouse is reported to be rapid during which time the compact and massive gland is reduced to a collection of small thin ducts (7, 46). Regression of the tissue in this study was also demonstrated by the epithelial to nonepithelial ratio. In the involuted gland, the ducts were longer and more ramified than in the unmated mouse.

Not only are these trends observed, but agreement with

previously published nonquantitative descriptions of pregnancy, lactation and involution occurred in this study.

Quantitative Study of Normal Tissue. The differences between the means obtained with 4 sq/in and 8 sq/in grids were slight and in no case did they alter the statistical significance of the differences between groups. Therefore, the 4 sq/in grid was considered an adequate tool to achieve the objectives of this study.

HAN and Spontaneous Tumor.

In this study, there seemed to be similarities between the previously described HAN and the spontaneously occurring tumor. What initially seemed to be hyperplasia, i.e. abnormal multiplication of normal cells in normal arrangement, appeared to have been morphologically equivalent to the well described HAN. Three distinct lines of evidence have been presented by other investigators to indicate the precancerous nature of the HAN: (1) HANs occur more frequently in high than in low tumor strains of mice, (2) histological transitions can be found between HANs and true mammary carcinomas, and (3) a certain percent grow progressively when transplanted, forming genuine malignant tumors (4, 5, 17, 18, 21, 33, 44, 47). The ability of the spontaneous nodule of this study to be passaged and grow when transplanted further suggested it had carcinogenic properties. Benign nodules do not have the ability to survive when transplanted more than once (17). The spontaneous tumor in this study has been passaged more than six times and continued to grow.

The progression of the HAN towards neoplasia required differentiation of epithelial tissue into two categories, alveolar and ductal. Although this further differentiation exceeds the scope of this study, such a recalculation would provide additional parameters of comparison between the HAN and the spontaneous hyperplastic lesion.

The relatively uniform distribution of the blood supply within the spontaneous tumor probably accounted for the absence of necrosis. The initial rate of growth in the spontaneous tumor was slow, the cells were well differentiated, and few mitotic figures were observed.

The exogenous tissue (H 2712) already established as cancerous had a decrease in the fraction of epithelial cells. An increase in necrosis was observed. Centrinodular necrosis occurred and increased with growth and the blood vessels appeared to be confined. In a previous study, McCredie injected ^{51}chromium-labeled erythrocytes into the mammary gland area and found that the red blood cells rarely entered the vessels in the area of necrosis. He concluded poor blood flow as therefore mainly responsible for cell death (36). The findings in this study suggest at least a coexistence of necrosis and poor blood supply. A higher percentage of undifferentiated cells was observed with subsequent passaging.

One of the difficulties in evaluating the sections of advanced exogenous tumors was distinguishing necrosis, edema, invasive connective tissue, and giant cells. As the tumor became more advanced, the cells lost their adherence to one

another, broke away and connective tissue invaded the area usually accompanied by edema. As the tumor's growth continued, its blood supply decreased and necrosis was observed.

The comparisons between the epithelial/nonepithelial ratios of any normal tissue to any tumor tissue indicated a statistically significant and higher ratio in tumor tissue. This attaches a value to the epithelial/nonepithelial ratio as a characteristic of tumor tissue. This is illustrated in Tables I - III. As can be observed from these tables, the nonoverlap of values allows for a nonparametric test for statistical significance.

Conclusion

The most important conclusions to be drawn from this study included:

1. The spontaneous mammary tumor behaved morphologically and functionally characteristic of HAN. It may represent an identical lesion.

2. The epithelial/nonepithelial ratios as developed in this study were a valid measure of normal mammary activity in that the data agreed with previously published histologic studies and additionally, provided an objective quantitative basis for comparison between functional states.

3. While the spontaneous and exogenous tumors initially exhibited significant differences on both a morphologic and quantitative basis, these differences decreased with each successive passage.

4. The epithelial/nonepithelial ratio begins to fail as necrosis represented a significant portion of the tumor tissue.

Extensions of this study could be projected in at least three directions: (1) further breakdown of epithelial tissue into alveoli and ducts to further establish the connection between HAN and the spontaneous lesion; (2) quantitation of blood supply using the same model and techniques to further establish the relationship between necrosis and blood supply; and (3) the use of an electronic scanner to perform the same analysis done manually in this study.

SUMMARY

A qualitative and quantitative histological study was made to correlate the architecture of normal mouse mammary gland tissue to spontaneous and exogenous mammary tumors. For this investigation, two high tumor strains of mice were selected. A quantitative tool, a ratio of epithelial/nonepithelial tissue as measured discretely on a 4 sq/in grid superimposed over hematoxylin and eosin stained microscopic tissue sections, was developed and the following points were established:

1. It can be quantitatively shown that variation in functional states occurs within normal mammary tissue; and

2. It can be quantitatively shown that differences occur between normal, hyperplastic and neoplastic tissue.

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Table 1

The nine categories of mammary tissue studied:

1. inactive
2. pregnant
3. lactating
4. involuting
5. spontaneous tumor
6. first passage spontaneous tumor
7. second passage spontaneous tumor
8. first passage syngeneic exogenous tumor (H 2712)
9. third passage exogenous tumor (H 2712)

Table 2

Epithelial	Nonepithelial	Necrosis (only in tumor tissue)
alveoli	blood vessels	any necrotic elements
ducts	connective tissue	
lumen	fat cells	
	lumen	

Table 3. Normal Mammary Tissue (Data for Figure I).

	INACTIVE			PREGNANT			LACTATION			INVOLUTION		
4 sq/in:												
Fat	378	376	381	294	287	285	136	149	132	358	325	340
Blood vessels	1	0	3	1	1	0	0	2	0	0	2	9
Epithelial	43	49	41	128	126	144	290	274	294	67	97	72
Total	425	425	425	423	414	429	426	425	426	425	424	421
Epithelial / Nonepithelial	0.11	0.13	0.11	0.43	0.43	0.5	2.13	1.8	2.23	0.18	0.29	0.21
Mean		0.12			0.46			2.05			0.23	
8 sq/in:												
Fat	892	918	898	696	694	707	360	415	388	848	796	865
Blood vessels	6	4	4	2	2	0	0	3	0	0	3	22
Epithelial	96	89	91	304	290	309	586	596	665	148	206	122
Total	984	1011	993	1002	986	1016	946	1014	1053	996	965	1009
Epithelial / Nonepithelial	0.11	0.1	0.1	0.44	0.42	0.44	1.63	1.43	1.71	0.17	0.25	0.14
Mean		0.1			0.43			1.59			0.187	

Table 4. Spontaneous Mammary Tissue (Data for Figure II).

	SPONTANEOUS BIOPSY			SPONTANEOUS - FIRST PASSAGE			SPONTANEOUS - SECOND PASSAGE		
4 sq/in									
Epithelial	377	370	365	383	390	390	361	354	342
Nonepithelial	23	30	35	17	10	10	39	46	58
Total	400	400	400	400	400	400	400	400	400
$\frac{\text{Epithelial}}{\text{Nonepithelial}}$	16.39	12.33	10.43	22.53	39.0	39.0	9.26	7.70	5.9
Mean		13.05			33.51			7.62	
8 sq/in									
Epithelial	938	923	912	965	974	974	915	919	859
Nonepithelial	62	77	88	35	26	26	85	81	141
$\frac{\text{Epithelial}}{\text{Nonepithelial}}$	15.13	11.99	10.36	27.57	37.46	37.46	10.76	11.35	6.09
Mean		12.49			34.16			9.40	

Table 5. Exogenous Mammary Tumor (Data for Figure II).

	EXOGENOUS -- FIRST PASSAGE			EXOGENOUS - THIRD PASSAGE		
4 sq/in						
Epithelial	311	318	361	300	346	320
Nonepithelial	46	49	39	67	54	60
Total	357	367	400	367	400	380
Necrotic	43	33	0	33	0	20
$\frac{\text{Epithelial}}{\text{Nonepithelial}}$	6.76	6.49	9.25	4.47	6.41	5.33
Mean		7.50			5.40	
8 sq/in						
Epithelial	829	868	935	764	911	813
Nonepithelial	82	94	65	144	89	111
Total	911	962	1000	908	1000	924
Necrotic	89	38	0	92	0	76
$\frac{\text{Epithelial}}{\text{Nonepithelial}}$	10.11	9.23	14.33	5.31	10.23	7.32
Mean		11.22			7.62	

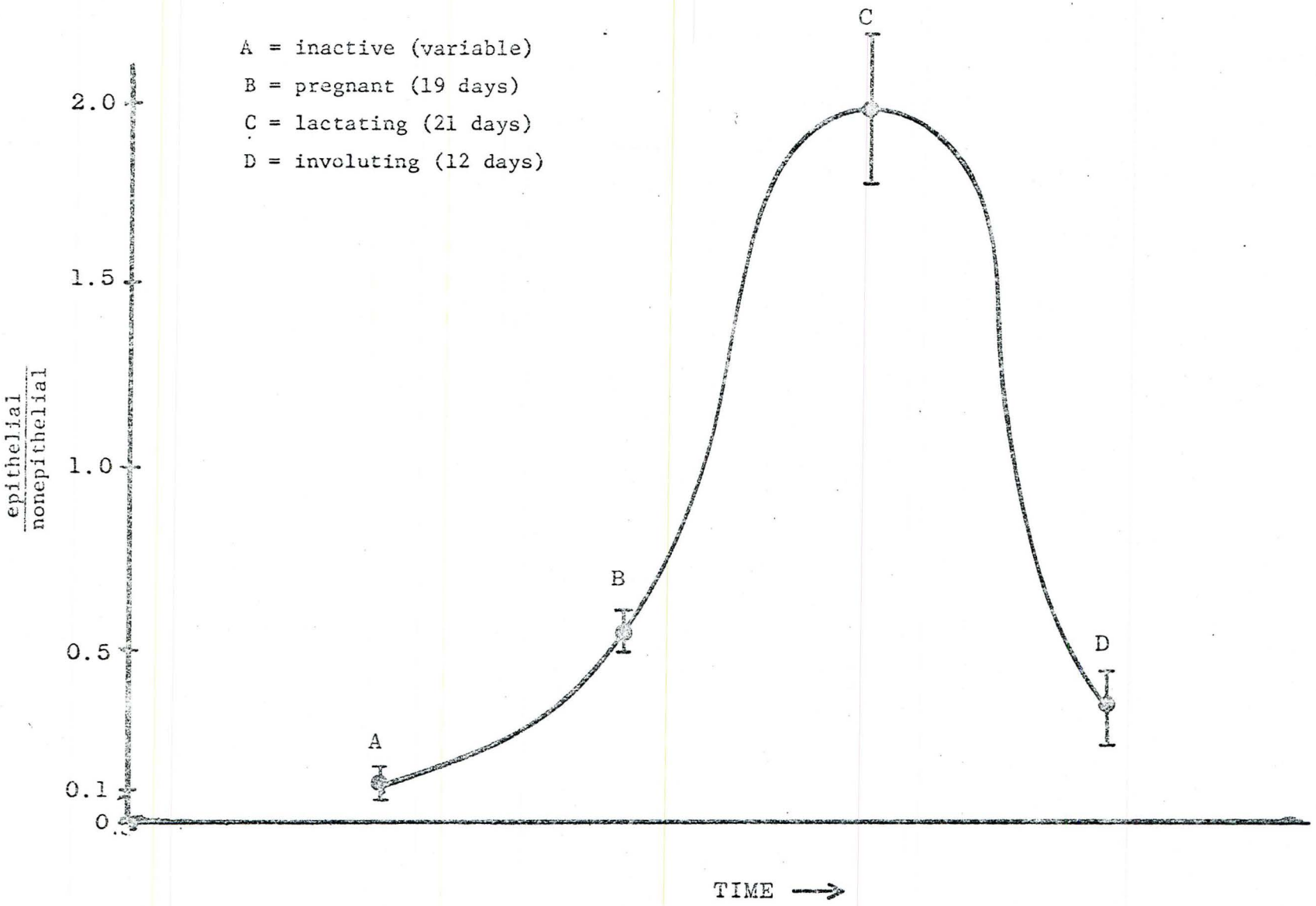
A = inactive (variable)
B = pregnant (19 days)
C = lactating (21 days)
D = involuting (12 days)
2.0
1.5
1.0
0.5
0.1
0
epithelial / nonepithelial
A
B
C
D
TIME

Figure I

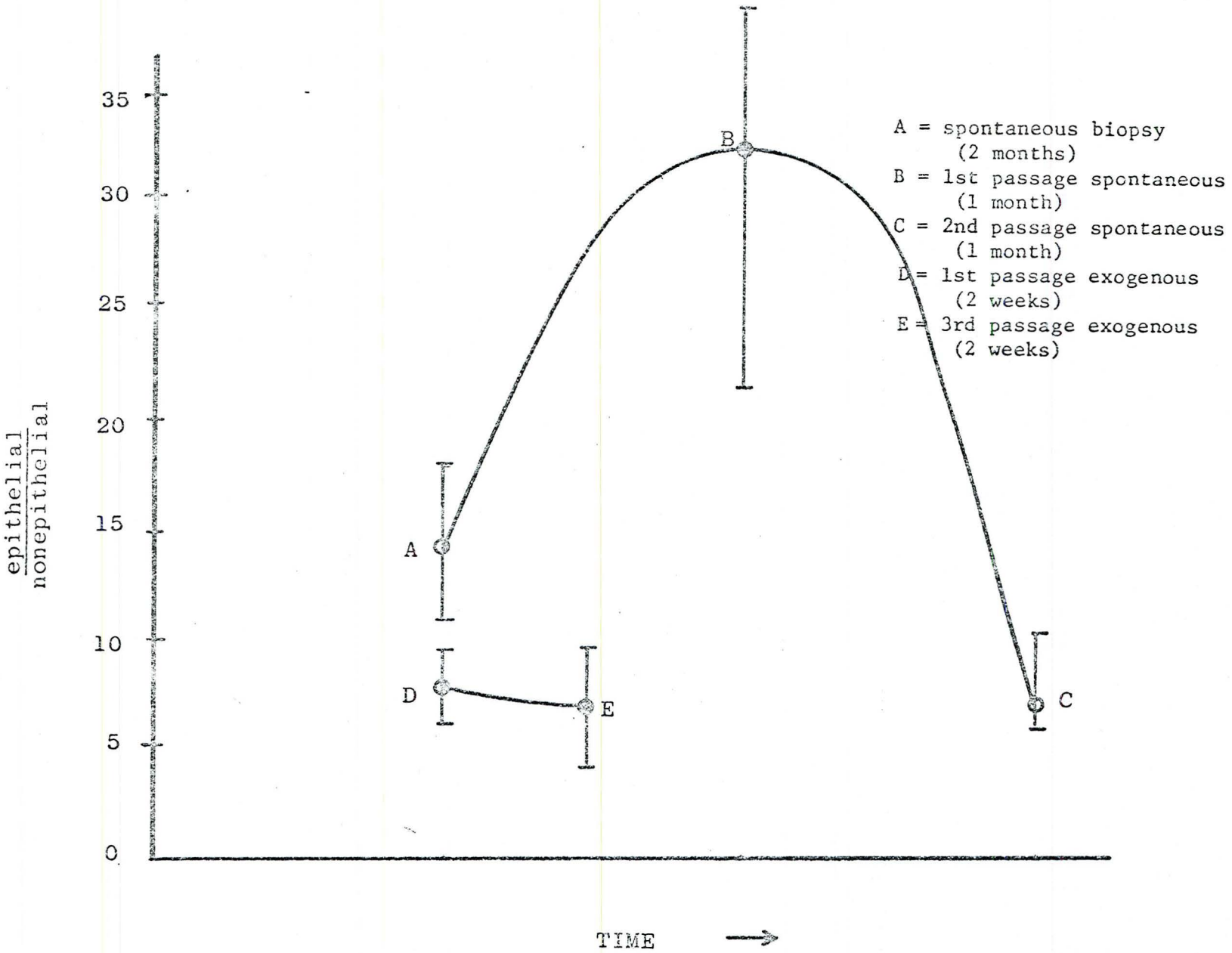
35
30
25
20
15
10
5
0
epithelial
nonepithelial
A
B
C
D
E
A = spontaneous biopsy (2 months)
B = 1st passage spontaneous (1 month)
C = 2nd passage spontaneous (1 month)
D = 1st passage exogenous (2 weeks)
E = 3rd passage exogenous (2 weeks)
TIME

Figure II

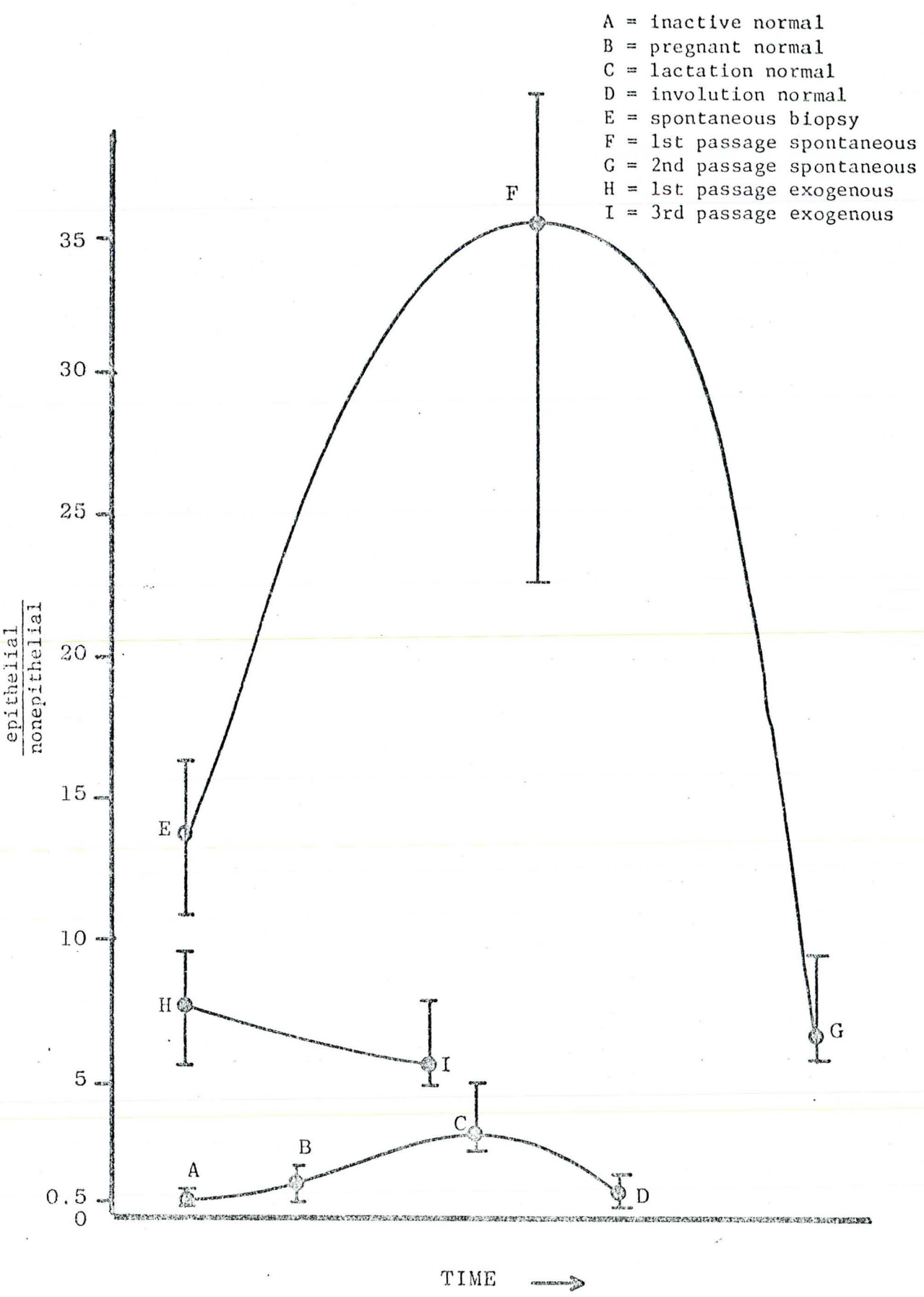
A = inactive normal
B = pregnant normal
C = lactation normal
D = involution normal
E = spontaneous biopsy
F = 1st passage spontaneous
G = 2nd passage spontaneous
H = 1st passage exogenous
I = 3rd passage exogenous
epithelial / nonepithelial
35
30
25
20
15
10
5
0.5
0
TIME

Figure III

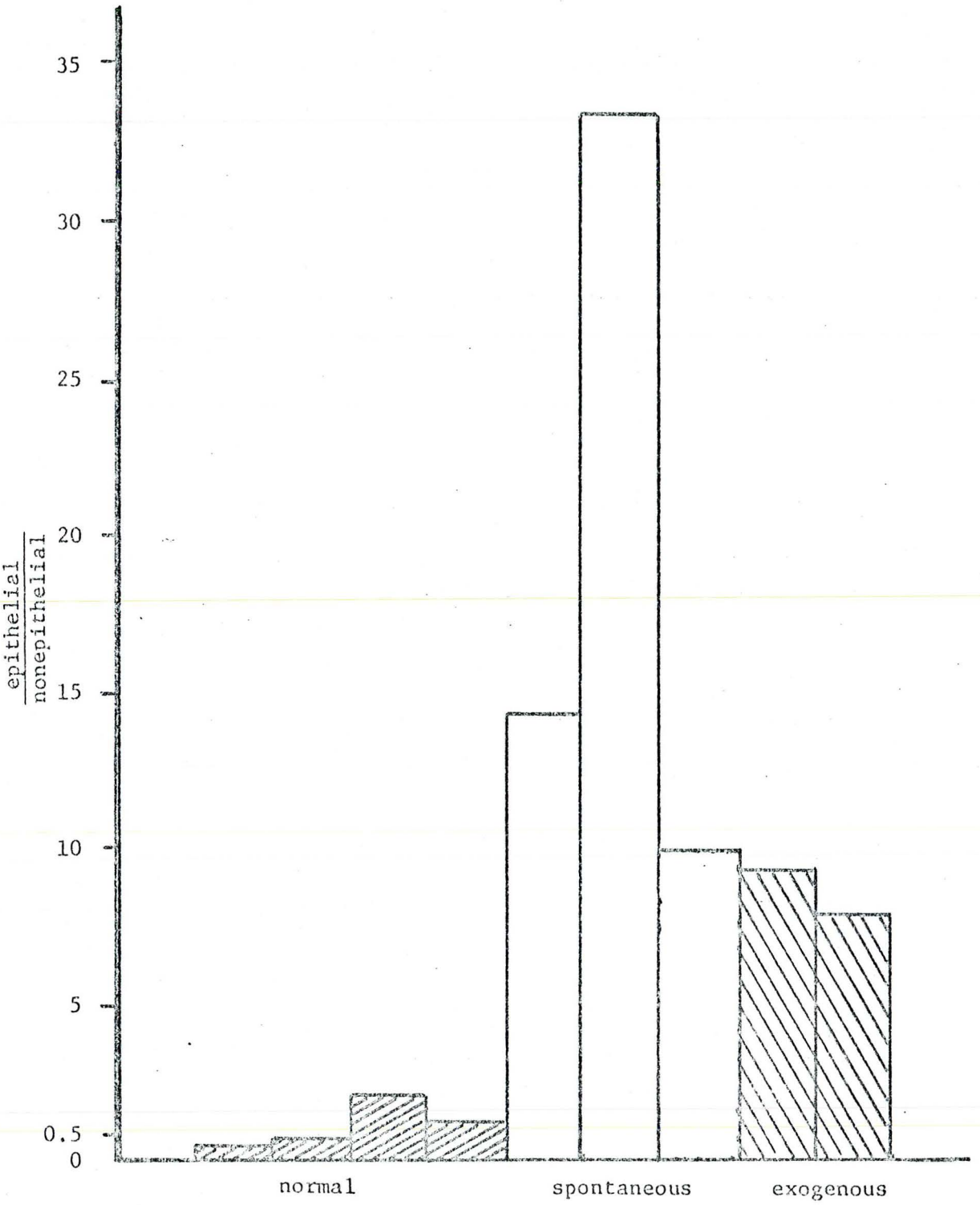
35
30
25
20
15
10
5
0.5
0
epithelial / nonepithelial
normal
spontaneous
exogenous

Figure IV

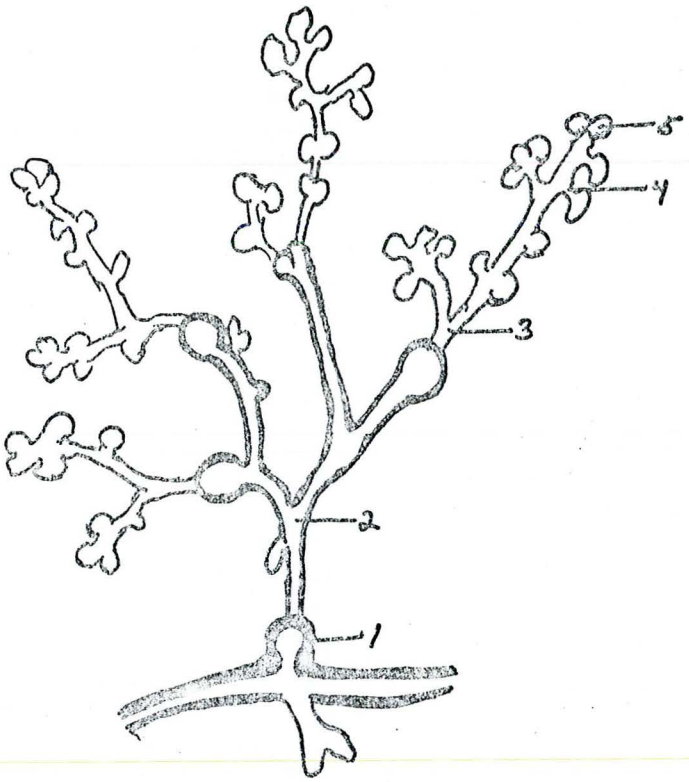

Figure A.

1. The anlage of the lobe appears as a bud-like outgrowth on the lateral wall of the duct. The bud is composed of a mass of epithelial cells. A lumen is formed coincident to the growth in length of the bud resulting in the formation of an intralobar duct. 2. Interlobular ducts derived from the lateral bud outgrowth, showing secondary buds at the lateral walls and terminal ends of the ducts. 3. Intralobar ducts derived from the lateral and terminal buds of the interlobular ducts. Multiplicity of the buds represents the true alveoli. 4. Intercallary duct, short duct establishing communication of the lumen of the alveoli to the intra- and interlobular ducts. 5. Alveoli.

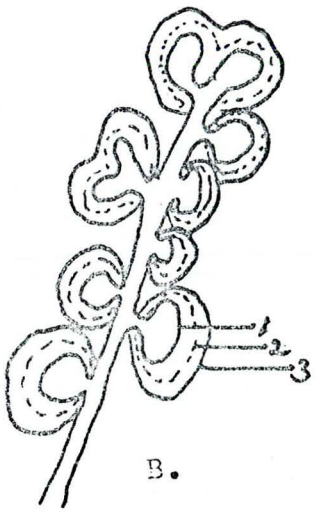

Figure B.

Shows direction of growth of the alveoli. 1. Alveoli in section of a 12 day old pregnant female. 2. Boundary of the alveolar wall (15th day pregnancy) as lumen begins to fill with secretion and 3. further distention of the alveoli and duct at parturition (46).

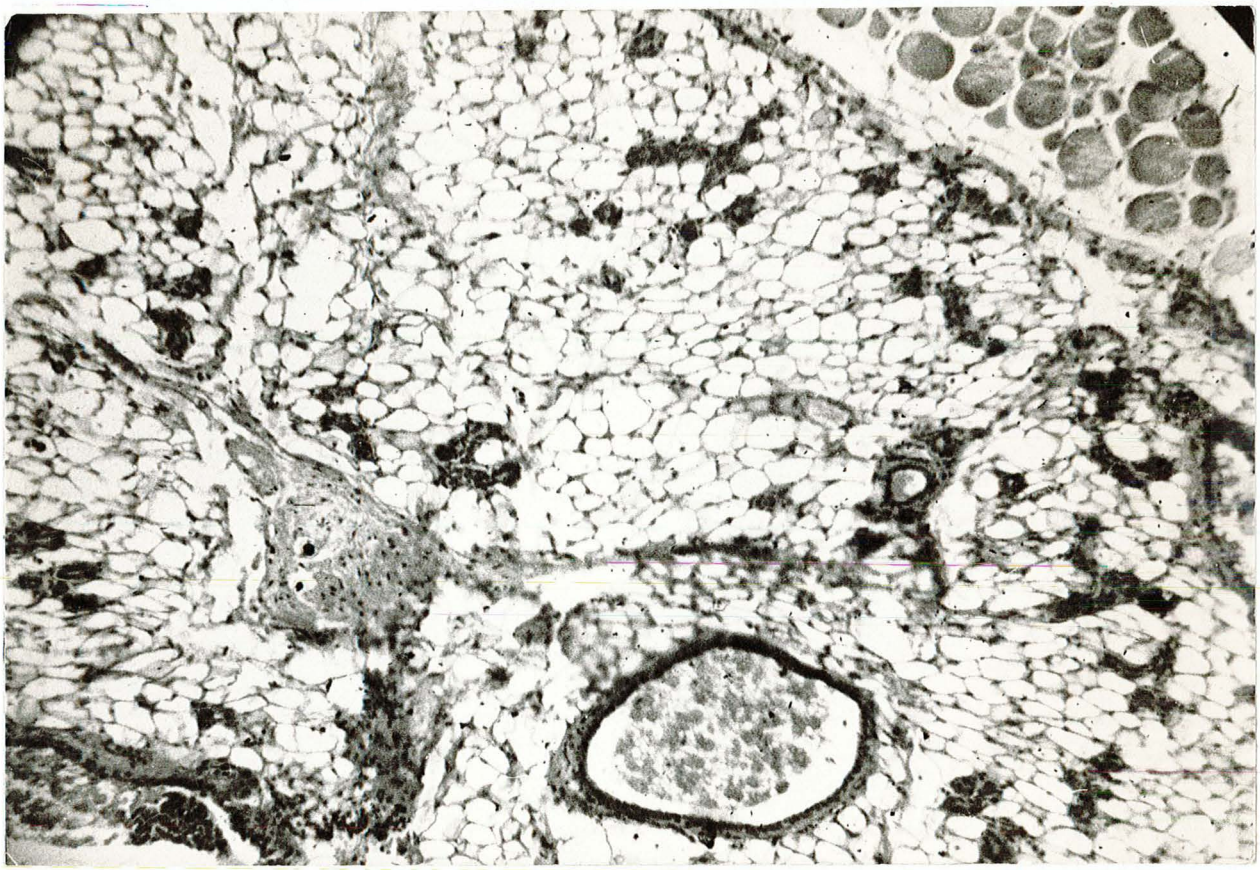

Plate 1. Inactive mammary tissue (10 X).

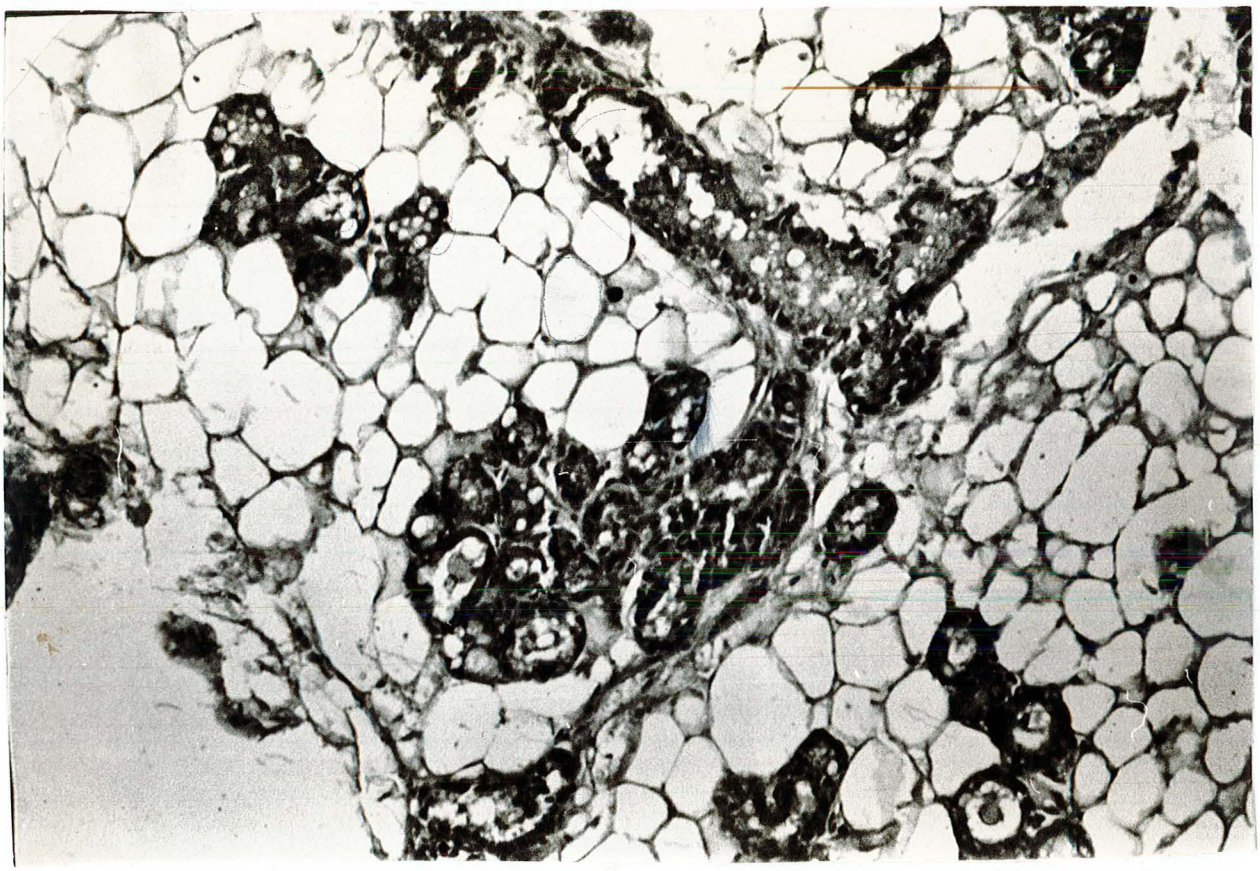

Plate 2. Pregnant mammary tissue (10 X).

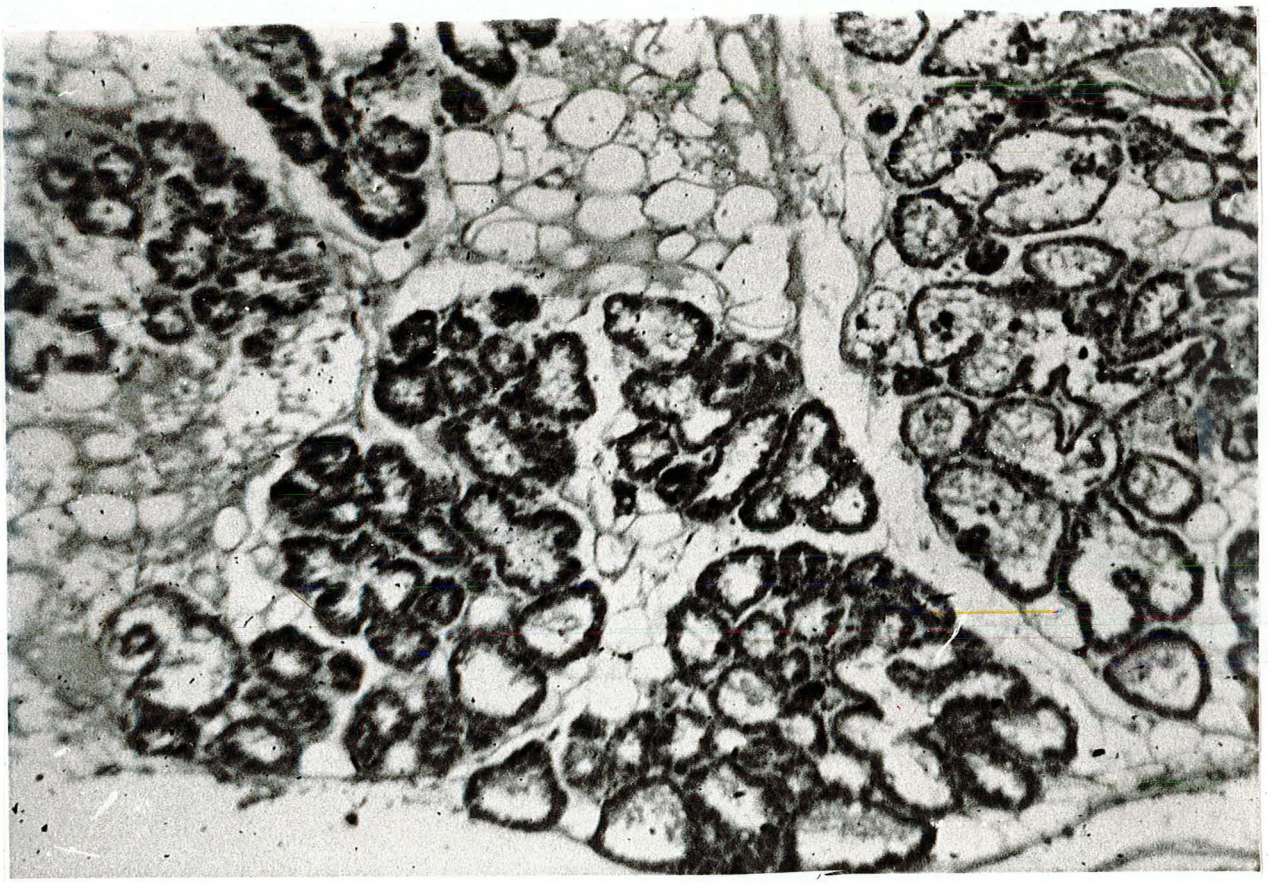

Plate 3. Lactating mammary tissue (10 X).

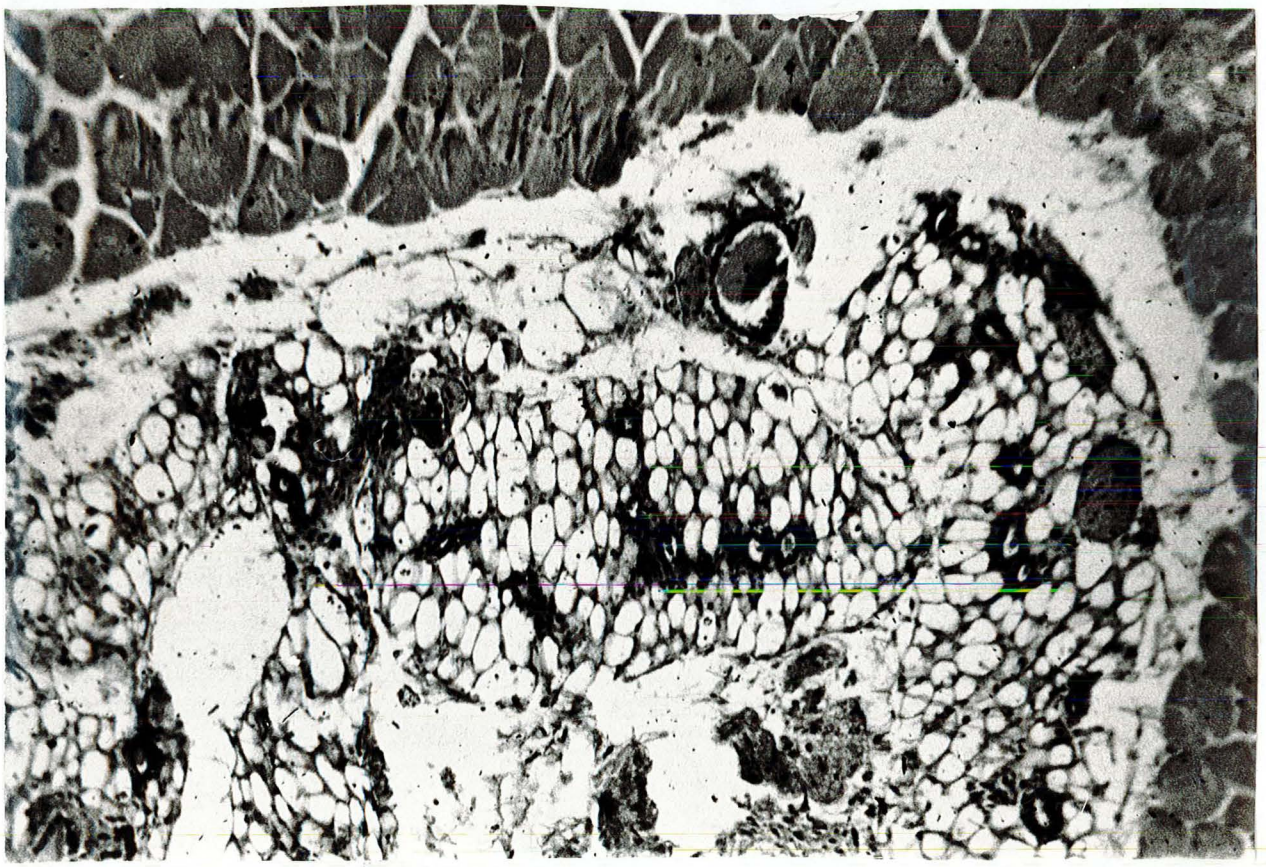

Plate 4. Involuting mammary tissue (10 X).

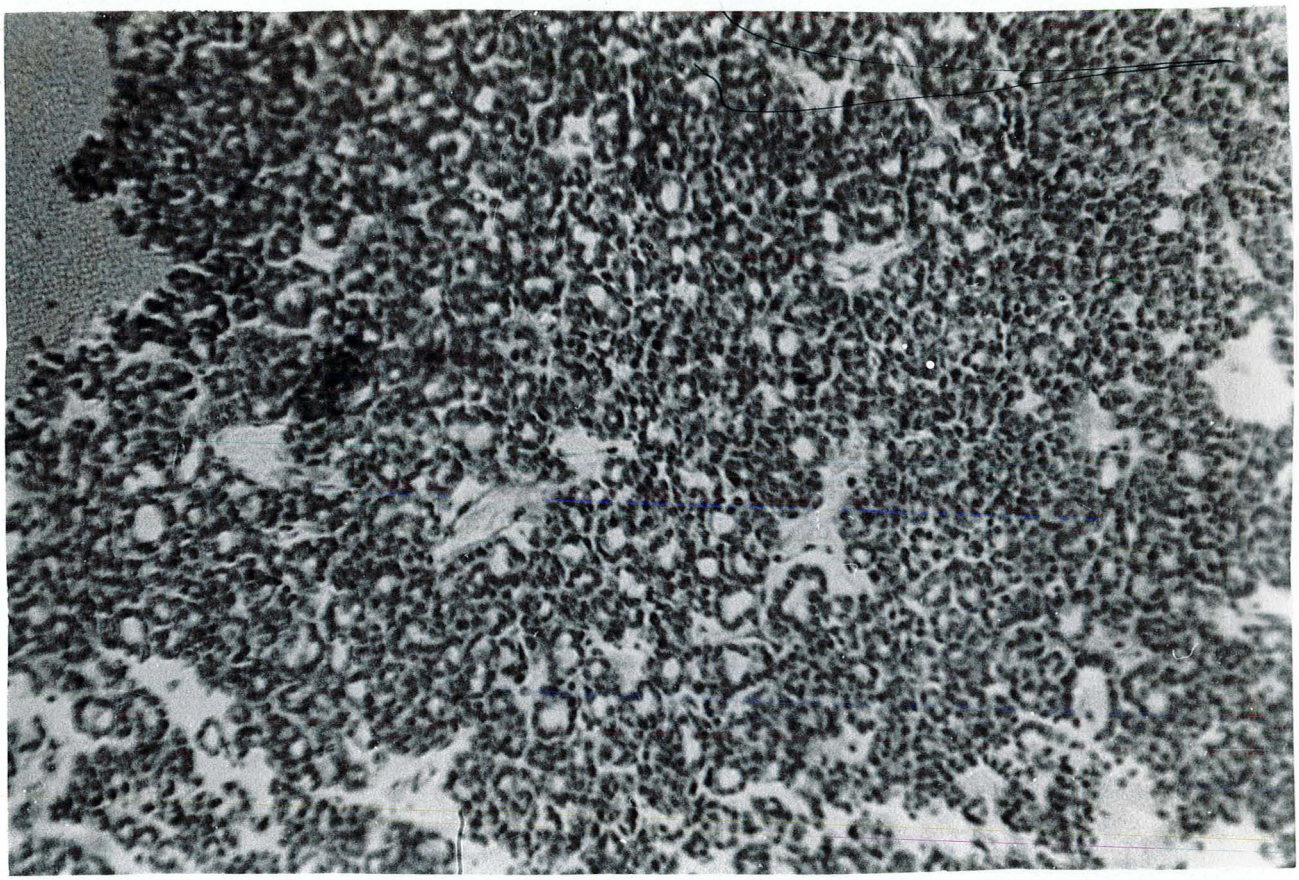

Plate 5. Spontaneous tumor (10 X).

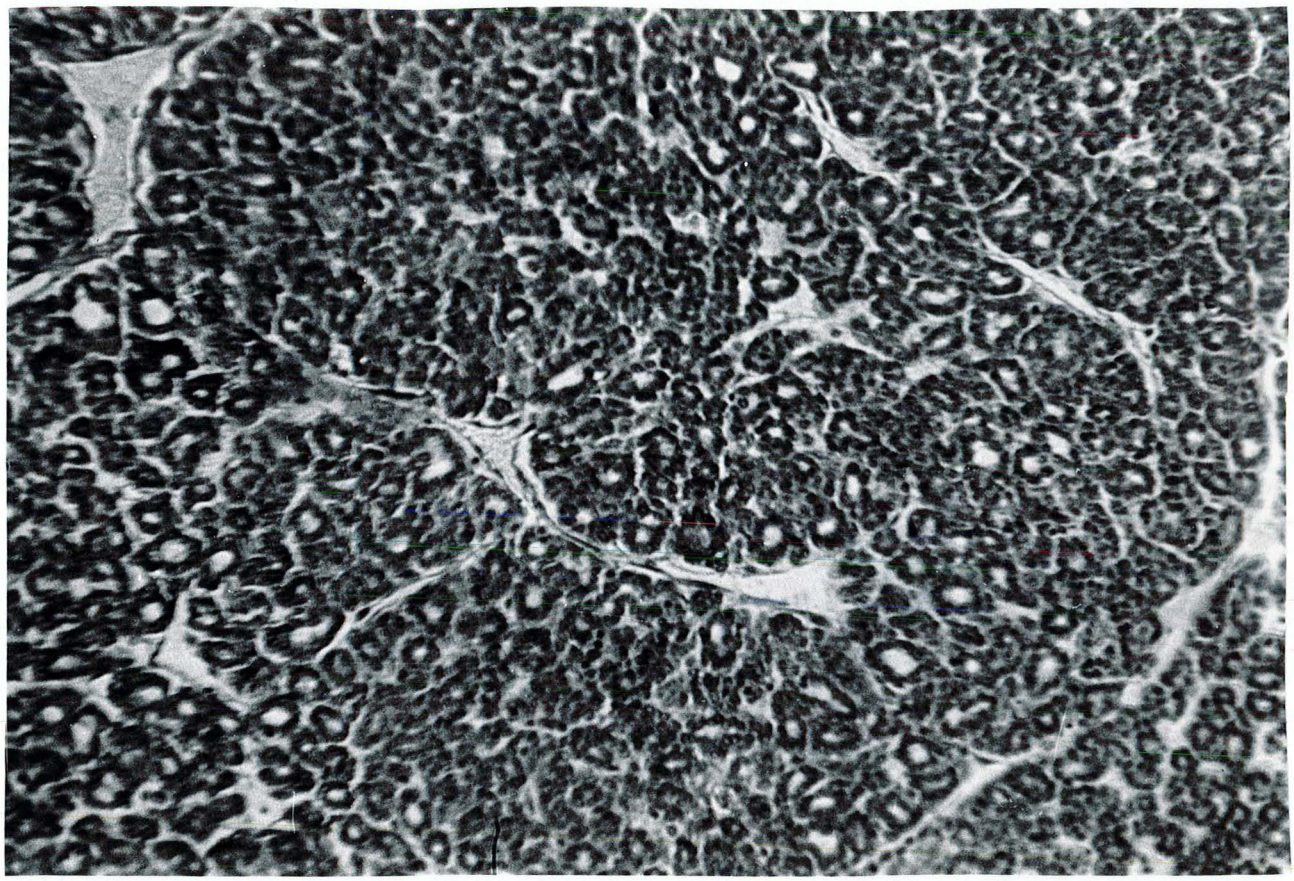

Plate 6. First passage spontaneous tumor (10 X).

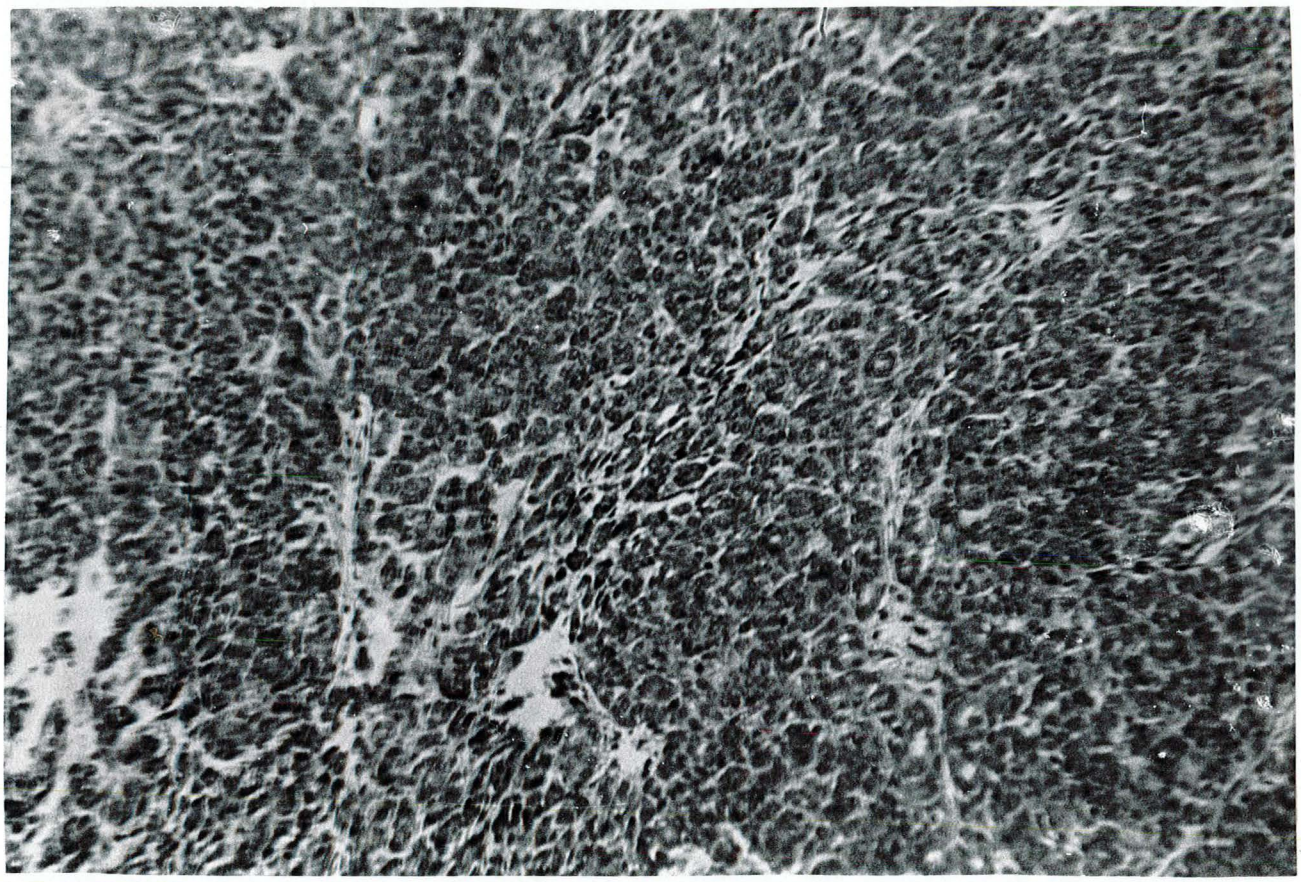

Plate 7. Second passage spontaneous tumor (10 X).

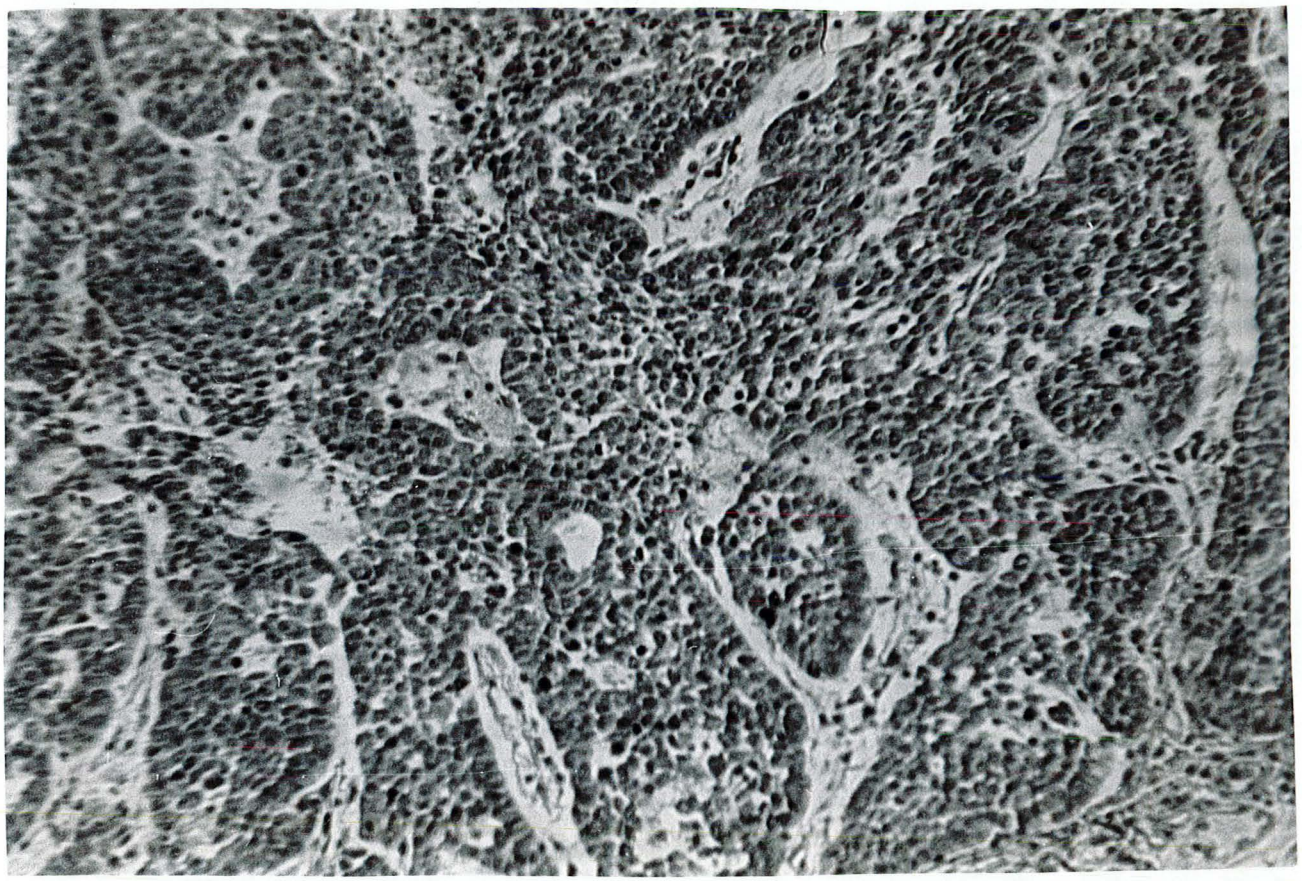

Plate 8. First passage exogenous tumor (H 2712) (10 X).

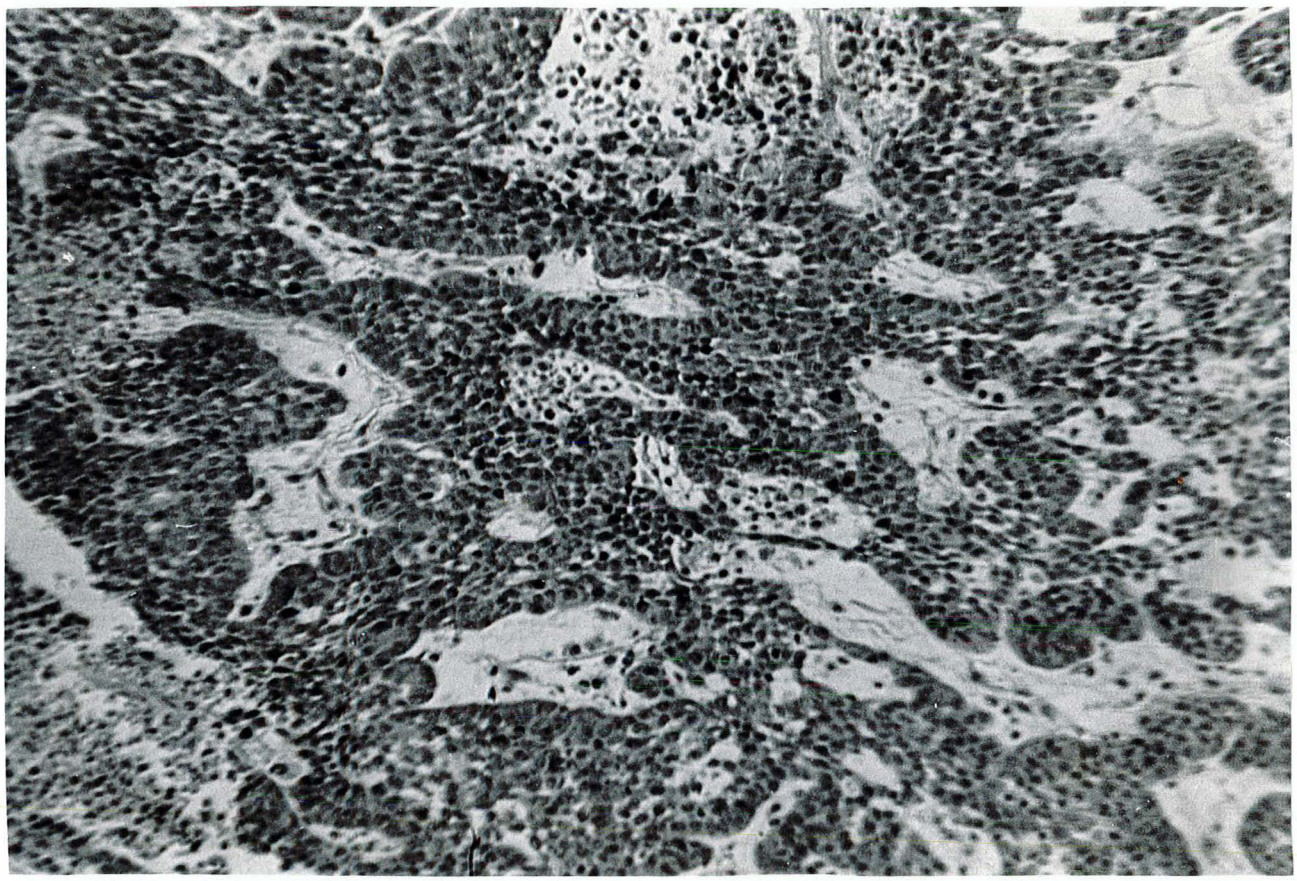

Plate 9. Third passage exogenous tumor (H 2712) (10 X).

ADDENDUM I

The Fisher Exact Probability Test: $Group_1$ vs. $Group_2$

	G_1	G_2	
above	a	b	A
below	c	d	B
	C	D	N

$$\frac{A!\ B!\ C!\ D!}{N!\ a!\ b!\ c!\ d!}$$

therefore:

	G_1	G_2	
above median	0	3	3
below median	3	0	3
	3	3	6

$$\frac{3!\ 3!\ 3!\ 3!}{6!\ 0!\ 3!\ 0!\ 3!} = \frac{3!\ 3!}{6!} =$$

$$\frac{3.2 \quad 3.2}{6.5.4.3.2} = \frac{1}{20} = 0.05$$